'IN VIVO' ROLE OF 'PSEUDOMONAS AERUGINOSA' TOXINS AND HOST RESPONSE

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Prepared for:

Office of Naval Research

15 January 1974

Security Classification

AD-773549

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1 ORIGINATING ACTIVITY (Corporate author)
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2a. REPORT SECURITY CLASSIFICATION
Unclassified

2b. GROUP

3 REPORT TITLE
In vivo role of Pseudomonas aeruginosa toxins and host response.

4 DESCRIPTIVE NOTES (Type of report and, inclusive dates)
Final Report (3-1-71 to 12-31-73)

5 AUTHOR(S) (First name, middle initial, last name)
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6 REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
January 15, 1974	~~7~~ 11	

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
N-00014-69-A-0235-0002	7
b. PROJECT NO NR 136-893	
c.	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d.	

10. DISTRIBUTION STATEMENT
Contractor-Imposed Distribution Statement Distribution of this document is unlimited.

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	Office of Naval Research

13. ABSTRACT

A protease from P. aeruginosa having collagenase activity exhibited lethal and dermonecrotic properties. It was purified 1500-fold and was capable of hydrolyzing collagen both in vitro and in vivo . The enzyme was assayed in mice by a number of routes and was found to be most toxic when administered into the lungs. This resulted in confluent pulmonary hemorrhage.

Five standard methods of extracting endotoxin were employed in an effort to establish its presence in Pseudomonas aeruginosa as well as to make comparative evaluations of its biological and chemical properties. Of the five preparations, aqueous phenol extracted endotoxin exhibited the greatest degree of lethality. The LD_{50} was 450 µg dry weight when administered intravenously and 840 µg intraperitoneally. No lethality was observed when endotoxin was administered intranasally. Lethality appeared to be associated with the core region of the lipopolysaccharide molecule, while no correlation between lethality and lipid content was detected. From the present studies, it appears that endotoxin from P. aeruginosa does not play a major role in the infectious process.

DD FORM 1 NOV 68 1473 (PAGE 1)

S/N 0101-807-6811

Security Classification

A-31408

14 KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Endotoxin from Pseudomonas aeruginosa						
Collagenase from P. aeruginosa						
Histopathology of P. aeruginosa infections						
Electron microscopy						

DD FOR 1 NOV 65 1473 (BACK)

S/N 0101-807-6801

A-31409

FINAL REPORT

OFFICE OF NAVAL RESEARCH

Contract N00014-69-A-0235-0002

Task No. NR 136-893

"*In Vivo* Role of *Pseudomonas aeruginosa* Toxins and Host Response"

by

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January 15, 1974

*The contents of this report have been submitted for publication and are *in press*.

"Summary of All Research"

A protease from *P. aeruginosa* having lethal and dermonecrotic properties, and capable of hydrolyzing native collagen, was purified 1,568-fold with a recovery of 24% by use of chemical and chromatographic technics. The enzyme preparation appeared to be homogeneous when subjected to chromatographic, electrophoretic, ultracentrifugational, and amino acid analyses. A standard state sedimentation coefficient of 2.10 S was calculated and further analyses indicated that the enzyme had a molecular weight of 17,500 and dimerizes under certain conditions to yield an apparent molecular weight of 34,000. In addition to native collagen, the enzyme catalyzed the hydrolysis of congocoll, azocoll, soluble collagen and casein, but did not attack orcein-elastin, azoalbumin, p-toluene sulfonyl-L-arginine methyl ester, benzoyl-L-tyrosine ethyl ester, and the hexapeptide N-Cbz-glycyl-L prolyl-glycyl-glycyl-L-prolyl-L-alanine. Enzymatic activity against congocoll was six-fold greater at pH 7.5 in Tris-HCl than in phosphate buffer at the same ionic strength. Cobalt, and to a lesser extent, zinc ions appeared to activate the enzyme, especially in phosphate buffer. Sodium cyanide and PCMB did not appreciably inhibit enzyme activity, while ammonium sulfate, EDTA and cystine displayed a significant inhibitory effect under certain conditions.

Several new areas of *in vivo* investigation using live cells of *P. aeruginosa* have been initiated. Preliminary studies indicate that mice receiving subcutaneous injections of the organism exhibit systemic infections after several days. The primary target organs appear to be the kidneys and lungs, with some pathology noted in other organs as well.

Administration of dead cells produced black necrotic lesions similar in appearance to ecthyma gangrenosum. Organisms administered into the lungs appeared to be rapidly cleared by the mice and death did not ensue unless the animal was previously debilitated by various chemical and physical treatments. However, per-oral administration of live organisms into the stomach resulted in an LD_{50} of 5×10^6 organisms. Other preliminary results with rabbits indicate that viable *P. aeruginosa* cells can induce disseminated intravascular coagulation (DIC), while dead cells or their endotoxin did not.

An extracellular protease from *Pseudomonas aeruginosa* having collagenase activity was assayed in vivo. The lethality of the enzyme for white female mice was determined by use of intravenous, intraperitoneal, intransal, and subcutaneous routes, respectively. The collagenase exhibited the following 72-hr mean lethal dose values: intranasally, 55 collagenase units; intraperitoneally, 148 collagenase units; and intravenously, 288 collagenase units. In the concentrations tested, no lethality was obtained when the subcutaneous route was employed. Gross and microscopic studies revealed that the collagenase was capable of eliciting a variety of tissue responses in mice depending upon its route of administraiton. Intranasal instillation resulted in confluent pulmonary hemorrhage, whereas intraperitoneal injections resulted in severe abdominal hemorrhage with foci on the intestinal serosa. Intravenous injections elicited abdominal hemorrhage and petechial hemorrhage with focal necrosis of the lungs, whereas subcutaneous injections resulted in necrotic, ulcerating lesions.

Attempts to obtain toxic preparations of endotoxin from *Pseudomonas aeruginosa* (C_9) were initiated by employing five different extraction procedures which were subsequently assayed for mouse lethality using a

variety of routes. The standard methods of extraction were the aqueous phenol, trichloracetic acid, ethylenediaminetetraacetate lysozyme, ethyl ether, and hot water procedures. The aqueous phenol preparation was found to be the most toxic and exhibited an LD_{50} value of 450 µg dry weight when administered intravenously and 840 µg intraperitoneally. No lethality was observed when endotoxin was administered intranasally. The second most lethal preparation was obtained by the trichloroacetic acid extraction and yielded LD_{50} values of 589 µg intravenously and 947 µg intraperitoneally. The other three preparations were considerably less lethal. The aqueous phenol and trichloroacetic acid preparation tended to have a higher content of those carbohydrates associated with the core region of the lipopolyssacharide molecule than did the lesser toxic preparations. Some correlation between lethality and lipid content as determined by the alkaline hydroxylamine procedure was also observed.

The endotoxin from *Pseudomonas aeruginosa* C_9 was obtained by five standard methods of extraction for comparative electron microscopic studies. Observation of the five preparations demonstrated the presence of two major types of structures. The first of these was seen in endotoxins prepared by trichloroacetic acid, ethyl ether, hot water, and ethyl-enediaminetetraacetate-lysozyme extraction, and consisted of discrete spherules containing smaller spherules within and having a homogeneous staining centre and rod-like border. The other morphologic type was seen only in preparations obtained by the aqueous phenol technique and consisted of pleomorphic staining material and rodlets. Preparations isolated by trichloroacetic acid extraction could be morphologically converted to the same appearance as aqueous phenol preparations by phenol extraction. Loss in structural integrity was encountered upon exposure to polymyxin B,

colistin sulfate and carbenicillin, but not with other antibiotics tested.

Chronic systemic infections of rabbits were established by intravenous inoculation of 4×10^8 *P. aeruginosa* cells in order to study the sequence of events leading to severe kidney damage. Renal lesions were detected by the 5th to 7th post-infection as were lesions in the liver and lungs. Progressive azotemia, determined by rising SUN and serum creatinine levels, led to death by the 12th to 16th day. Lesions in the kidneys, lungs and liver were characterized terminally by intense mononuclear cell infiltrates, hemorrhage, and microabscess formation. Mononuclear cells appeared to be the predominant responsive cell early in infection. In addition, there appeared to be no difference in susceptibility to infection or severity of renal lesions between rabbits with surgically induced unilateral ureteral obstruction and non-ligated rabbits.

Preparative technics were developed for the purpose of visualizing, by negative contrast, the internal structures of *Pseudomonas aeruginosa*. Some of the technics employed treatment of bacteria with detergents such as Biz, Punch, and Nonidet P-40. Rhapidosomes were found within uninduced cultures and appeared to be concentric double cylinders, usually oriented in adjacent pairs, and spatially arranged in skewed as well as in parallel alignment with the longitudinal axis of the organisms. The mean dimension of the major cylinders were 23-27 nm x 237 nm, and the inner cylinders appeared to be 6.7 nm in diameter. Within the inner cylinder a twisted ribbon-like structure with a helical periodicity of 55 nm was seen. A very thin envelope or film was occasionally seen at the periphery of the rhapidosomes, especially around the curvature of an unbroken end

Rhapidosomes appeared visible in about 25% of the electron transparent population on the grids. The maximum number of these microtubule-like structures detected per cell was seventeen, although the average number per cell ranged from four to twelve. Unlike pyocin, the bacteriocin of pseudomonads, neither contracted microtubules nor tail fibers were detected. The minimal nutritional requirements for the production of rhapidosomes as well as detailed purification procedures are described.

2. Index of all technical reports

1) 6 month report, September 1, 1971

2) Annual report, March 1, 1972

3) 6 month report, September, 1972

4) Annual Report, March 1, 1973

5) 6 month report, September 1, 1973

6) Annual Report, December 31, 1973
Final Report, January 15, 1974

3. Index of All Publications:

a. *In vivo* studies with collagenase from *Pseudomonas aeruginosa*. Diener, B., Carrick, Jr., L., and Berk, R.S. Infection and Immunity, 7:212-217, 1973.

b. Comparative studies on *Pseudomonas aeruginosa* endotoxin. Dyke, J. and Berk, R.S. Z. f. Allgemeine Mikrobiologie, 13:307-313, 1973.

c. Comparative electron microscopic studies on *Pseudomonas aeruginosa* endotoxin. Dyke, J. and Berk, R.S. Z. f. Allgemeine Mikrobiologie, 13:381-393, 1973.

d. Electron microscopic examination and quantitation of rhapidosomes, viruses and bacteria concentrated at an aqueous-air interface. Baechler, C.A., Taylor, A.R. and Berk, R.S. Preparative Biochemistry, 2:287-296, 1972.

e. Ultrastructural observations on *Pseudomonas aeruginosa*. I. Rhapidosomes Microstructures, 3:24-31, 1972.

f. Electron microscopic observations of *Pseudomonas aeruginosa*. Baechler, C. and Berk, R.S. Z. f. Allgemeine Mikrobiologie, 14 (4), 1974 (in press).

g. Growth inhibition and pyocin receptor properties of endotoxin from *Pseudomonas aeruginosa*. Dyke, J. and Berk, R.S. Proc. Soc. Exp. Biol. Med. Submitted for publication.

h. Purification and characterization of collagenase from *P. aeruginosa*. Carrick, Jr., L. and Berk, R.S. Submitted to J. Biol. Chem.

i. Several other manuscripts are currently under preparation.

4. Conclusions

A number of interesting conclusions were made from this study. First, it was established that *Pseudomonas aeruginosa* produces a proteolytic enzyme which behaves like a true collagenase. The enzyme had properties and enzymatic activity comparable to the collagenases described in anaerobes such as *Clostridium perfringens* and related organisms. Secondly, it was established that the enzyme could be purified several thousand fold despite its extreme lability and that it behaved as a potential virulence factor when assayed *in vivo*. The collagenase was found to be both lethal and dermonecrotic and it was capable of destroying collagen and other substances in tissues when administered to normal mice. Third, endotoxin isolated by five different extraction procedures was found to vary in its biological activity depending on the method of extraction. Also, the toxicity of the most potent preparations were considerably less potent than endotoxin from other gram negative organisms. These results suggest that endotoxin from *P. aeruginosa* does not seem to play a major role in the disease process. Fourth, electron microscopic studies of the various endotoxin preparations indicate that their structure can be markedly altered by *in vitro* treatment with either polymyxin B, colistin

sulfate or Carbenicillin. These results suggest that the biological potency of these treated preparations could theoretically be increased or decreased by routine chemotherapy of infected patients. Fifth, studies on rabbits indicated that surgically ligated kidneys (unilaterally), did not predispose the animals to infection as compared to unligated, infected animals. Sixth, mice infected subcutaneously died from systemic infection with the lungs and kidneys being the major target organs. Dead cells were not lethal, but produced a black necrotic lesions resembling the clinical entity "erythyma gangrenosum." Finally, mice infected per-orally in the lungs were able to clear the organisms without untoward symptoms. The antineoplastic drug methotrexate was unable to pre-dispose these mice to pulmonary infection. On the other hand, per-oral infection of the stomach led to systemic infection and death with striking histo-pathological lesions in many internal organs.

5. List of Major Accomplishments

a) purification and *in vitro* characterization of a protease exhibiting collagenase activity from *P. aeruginosa.*

b) *In vivo* study of purified collagenase from *P. aeruginosa* leading to a gross and histopathological study. It was established that the collagenase is a potential virulence factor.

c) Extraction and characterization of endotoxin from *P. aeruginosa.* Endotoxin from these cells was consistently less toxic than endotoxin from other gram negative bacteria.

d) Pulmonary studies using viable cells indicated the mouse to be refractory to death by infection.

e) Orally infected mice were susceptible to infection which eventually became systemic.

f) Mice infected subcutaneously or intradermally died from systemic infection.